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Biopriming of Stevia rebaudiana seeds by plant growth promoting bacteria to improve seed germination

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Abstract: Poor germination and low seedling growth of Stevia rebaudiana Bertoni are common problems in the cultivation of the plant causing scarce plant material and high cost of the product. A consistent, efficient and eco-friendly approach could be the exploitation of biostimulants seedlings and plant-based biostimulants (PBs) as microbial, natural or organic-based, which may have the potential to stimulate plant growth in synergistically. Seed biopriming was performed by four plant growth promoting bacteria; Bacillus cereus, Bacillus licheniformis, Bacillus paralicheniformis and Bacillus paramycoids. Soaking of seeds in bacterial suspension of plant growth promoting strains and in co-culture between them overnight under aseptic condition proves that the significance of co-culture in the improvement of seed germination with percentage up to 40% compared with single strains of 20% and control of 5%. Therefore, we suggest that seed biopriming with the integrated application of these four biostimulants at an appropriate concentration can enhance the poor germination performance of S. rebaudiana and improves the seedling growth.

keywords: Seed priming; *Stevia*; Germination indices; Biostimulants; Endophytic colonization.

Introduction

Stevia rebaudiana is a perennial herb that belongs to the family Asteraceae. The interest of this plant has risen in recent years due to its content of steviol glycoside. Steviol glycosides are about 200 times sweeter but much less calorific than sugar **[1]**. It is considered as a good substitute for sugar as a healthy natural sugar substitute for diabetic patients. It is a zero calorie sweetener, indigestible in the human digestive tract, so it is not possible to break down the compounds chemically **[2]**. The problem with this plant caused difficulties to the large-scale establishment of *S. rebaudiana* because of poor germination of seeds-10% and 36.3% **[3, 4]**. Seeds are very small (1000 seeds weigh 0.3:1.0 g) and as a result seedlings are slow to develop, reaching a size suitable for transplanting to the field at 45-60 days revealed that some active manipulation of the blossoms is necessary to achieve pollination. Seed yields of up to 8.1 kg ha^{-1} have been recorded, but it is common to achieve less than 50% germination **[5]**. This resulted in the supply and cost-effectiveness of the plant materials becoming scarce. Poor rates of germination do not mean that the seeds are dead but environmental factors such as very low humidity and high temperatures and endogenous factors may be responsible for non-germinability in plants. However, some reports have explored the possibility of improving *S. rebaudiana* seed germination by focusing on such factors as temperature light and different seed treatment methods **[6]**. Microbes actively involved in crop production are commonly referred to plant growth promoting bacteria (PGPB), whereas bacteria isolated from the root zone are referred to as plant growth promoting rhizobacteria **[7]**. Endophytic bacteria live in plant tissues that are capable of nitrogen fixation, siderophore and IAA hormones synthesis and do not cause disease symptoms **[8]** . Endophytic microbes inhabitants of *S. rebaudiana* plant are mainly dominated by bacterial groups *Bacillus* spp., *Erwinia* spp, *Agrobacterium* spp. and *Methylobacterium* spp **[9]**. The dual ability of this microbial community is to be a biological agent that promotes plant growth and can also

improve plant resistance to pests and diseases. As a growth promoter, microbial effectiveness is due to its ability to generate growth hormones (IAA, gibberellin, and cytokines) that are naturally needed by plants to improve their growth and development **[10, 11]**. Application of PGPR by seed priming, soaking the seeds in liquid bacterial suspension for pre-measured time, starts the physiological processes inside the seed while preventing the emergence of radicle and plumule **[12]**. This study aims to use endophytic bacteria isolated from *S.rebaudiana* leaves and have plant growth promoting criteria that can effectively enhance the germination of *S.rebaudiana* seeds through seed biopriming.

2. Materials and methods

The present experiment was carried out at Plant Cytology and Genetic Laboratory, Botany Department, Faculty of Science, Mansoura University, Egypt in 2020. The protocol was developed to overcome poor seed germination in *Stevia rebaudiana* by PGPB application through seed priming, soaking the seeds for a time in a liquid bacterial suspension.

Studying the synergistic and antagonistic relation between endophytic bacteria isolated from *Stevia rebaudiana* leaves:

Endophytic bacteria isolated from *S. rebaudiana* were molecularly identified as *Bacillus cereus* SA1, *Bacillus licheniformis* SA2, *Bacillus paralicheniformis* SA3 and *Bacillus paramycoids* SA4, with accession numbers MW042692, MT066091, MW042693 and MT066092 respectively. L.B solid media plates were prepared and then inoculated with bacterial isolates to study synergism and antagonism. The microbes studied were spontaneously grown on the same plate, with cross-growth between each pair of endophytes of *S. rebaudiana* plant to test the presence of any apparent antagonism between them. Then the plates were incubated for 48 hr at 28^{o}C.

Surface sterilization of *Stevia rebaudiana* seeds

To conduct the germination test, first, all used equipment and seeds were thoroughly sterilized. The surface sterilization of *S. rebaudiana* seeds proceeded as follows: the seeds were first washed under a running tap. Then, the seeds were sterilized for 2 minutes with 70 % ethanol and then washed with distilled water three times. Besides, the same seeds were sterilized with Sodium hypochlorite solution 3% (NaOCl) for 5 minutes then were rinsed three times with sterile distilled water **[13]**.

The first trial of applying the synergistic organisms on the seeds in single and co-culture treatments:

Under aseptic conditions, *S. rebaudiana* seeds were surface sterilized. Then, they were soaked into 50 ml of bacterial suspension with optical density at 600 nm =0.8 overnight with shaking and seeds soaked in distilled water as the control. Seeds were divided to two equal groups, the first group inoculated on containers contain sterilized moistened cotton and the other group inoculated and placed between double sterilized Whatman filter paper (No.1) in 12-cm-diameter Petri dishes moistened with sterilized-distilled water. To avoid possible primary dormancy phenomena, seeds incubated in dark conditions for 3 days for germination. Seeds were transferred to a germination cabinet adopting the same temperatures (25±2 ^{o}C) and light or dark conditions

The second trial on planting tray of the most promising isolates and co-cultures on *Stevia* seeds:

S. rebaudiana seeds were sterilized according to the previous procedures. Then, they were soaked into 50 ml of bacterial suspension with optical density equal 0.8 at 600 nm overnight with shaking; while control seeds soaked in distilled water. In order to improve the growth, treatments with the best germination ratio of the first trial were qualified to this trial. 20 seeds for each treatment were then distributed in a planting tray filled with peat moss as the sowing medium. At a temperature range of 26-29^{o}C for 7 days, the tray was left in natural day/night conditions. After that, 180 primed, unprimed (control) seeds were counted, and germination percentages were then calculated as follow:

(i) Final germination percentage (FGP) = (number of germinated seeds/total number of sown seeds) x 100%.

(ii) Mean daily germination (MDG) =Final germination percentage / number of days to final germination

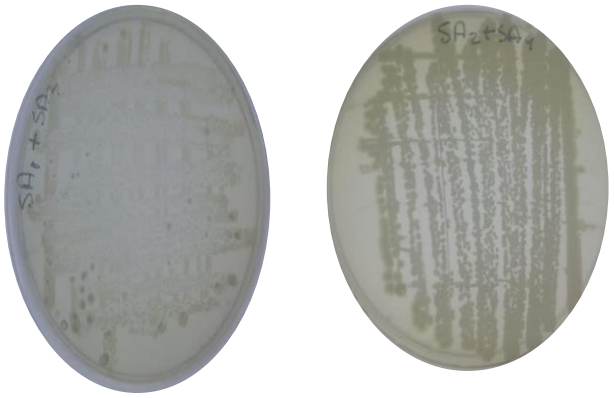
(iii) Germination value (GV)= MDGXFGP

According to **[14]**, **[15]** and **[16]** respectively.

3. Results and Discussion

The synergistic and antagonistic relation between isolated endophytic bacteria from *Stevia rebaudiana* leaves

The normal growth activity of the isolates confirms the failure of any potential antagonism between each pair of microorganisms examined. **Figure.1** represents the result on petri dishes where **A.** represents synergism as *Bacillus cereus* SA1 and *Bacillus paralicheniformis* SA3 can growth normally together while **B.** antagonism as *Bacillus paramycoids* SA4 prevent *Bacillus licheniformis* SA2 grow normally over the media, all results described in **Table 1**, Green colour represent synergism and red colour represent antagonism.



A) **B)**

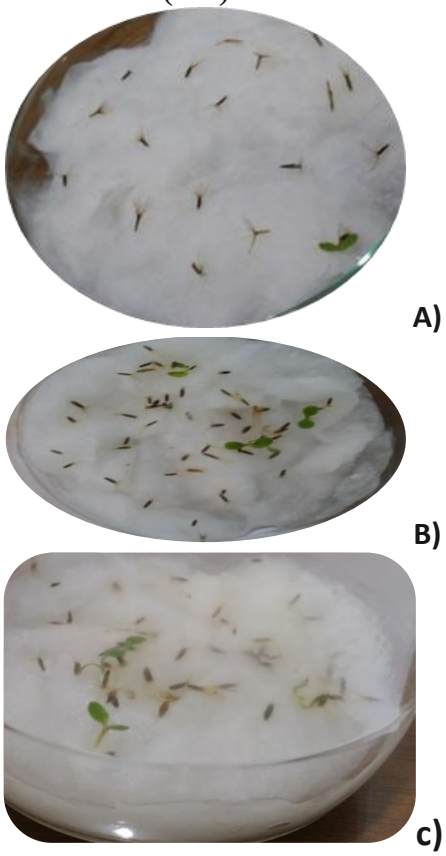
Figure1. Representative result of interaction between strains on petri dishes where **A.** represent synergism while **B.** antagonism

First trial of biopriming of seeds by bacterial isolates in single strains and synergistic co-culture:

The resulted synergistic bacteria used in applying seeds biopriming and inoculation of seeds in petri dishes with filter paper and container with cotton. Six out of eight trials were germinated faster after 4 days (*Bacillus cereus* SA1, *Bacillus licheniformis* SA2, *Bacillus paralicheniformis* SA3, *Bacillus paramycoids* SA4, *Bacillus paralicheniformis* SA3+ *Bacillus paramycoids* SA4 and *Bacillus cereus* SA1 + *Bacillus paralicheniformis* SA3) compared with control which started in germination after 10 days and reported in **Figure 2.**

Second trial on planting tray contains peat moss and the most effective treatments compared with control:

The germination started from the fourth day of sowing in most treatments except control started on 10^{th} day. Co-culture between *Bacillus cereus* SA1 and *Bacillus paralicheniformis* SA3 was the most effective treatment with a germination percentage of 40 %, while co-culture between *B. paralicheniformis* SA3 and *B.paramycoids* SA4 germination percentage was 30%. In single strains, *B.cereus* SA1was 20%, *B. licheniformis* SA2 10%, *B.paralicheniformis* SA3 10% and *B.paramycoids* SA4 25% compared to control 5 % after 10 days of inoculation **(Figure 3)**. The summary, the rate and the percentage of seed germination in *S. rebaudiana* was successfully improved through biopriming of seed by plant growth promoting endophytic bacteria in single strain treatments and co-culture between different strains compared with control**. Table 3** illustrates the germination percentage (GP), Mean daily germination (MDG) and Germination value (GV).



A)

B)

c)

Figure.2 represents the germination percentage after 10 days of inoculation **A.** represent control while **B.** & **C.** represent treatments

Table.1. Synergistic and antagonistic relation between endophytic bacterial isolates

Isolates	***B. cereus* SA1**	***B.licheniformis* SA2**	***B.paralicheniformis* SA3**	***B.paramycoids* SA4**
B. cereus SA1				
B. licheniformis SA2				
B.paralicheniformis SA3				
B. paramycoids SA4				

Green colour represent synergism and red colour represent antagonism.

Figure 3. Representative of the fourth day of the second trial of seed germination in planting tray after biopriming.

Table 3.represents germination percentage (GP), Mean daily germination (MDG) and Germination value (GV) of the most significance treatments**.**

Treatment	**GP%**	**MDG**	**GV**
Control (unprimed)	5.00 ± 0.14^{a}	0.50 ± 0.03^{a}	2.50 ± 0.01^{a}
B. cereus SA1	20.00 ± 0.69^{c}	2.00 ± 0.12^{c}	40.00 ± 0.06^{c}
*B. icheniformis*SA2	10.00 ± 0.23^{b}	1.00 ± 0.04^{b}	10.00 ± 0.02^{b}
B.paralicheniformis SA3	10.00 ± 0.23^{b}	1.00 ± 0.04^{b}	10.00 ± 0.02^{b}
B.paramycoids SA4	25.00 ± 0.72^{d}	2.50 ± 0.13^{d}	62.50 ± 0.06^{d}
B. cereus SA1 + *B. paralicheniformis* SA3	40.00 ± 0.92^{f}	4.00 ± 0.16^{f}	160.00 ± 0.08^{f}
B.paralicheniformis SA3 + *B.paramycoids* SA4	30.00 ± 1.04^{e}	3.00 ± 0.18^{e}	90.00 ± 0.09^{e}

The results are recorded as Mean of triplicates ± Standard Error (S.E). Different superscript letters refer to significant differences ($P < 0.05$)

Discussion

S.rebaudiana plant reported having poor seeds germination not because they are seeds dead but due to environmental factors [17]. Seed priming is soaking the seeds in any solution containing certain necessary priming agents such bacterial suspension, except the development of the radicle, which results in

the initiation of the physiological and the germination process [12, 18]. Endophytic colonization involves bacterial penetration into the plant tissues, which shows the traits of plant growth promoting [19]. In this study plant growth promoting endophytic bacterium used in biopriming of seeds and combination between strains showed very effective improvement percentage of seeds germination, plant growth promoting strains produce bioactive compounds and hormonal stimulation which share in plant promotion and diseases defense [20]. Co culture produce new bioactive compound and secondary metabolites improve crop productivity and plant health [21] and these results agreed with Khan study of seed treatment[22] and the study of seed priming performed by [23]. The effect of the co-cultures of the endophytes in this study was significant as the percentage of germination was increased from 5% to 40% compared to control

Conclusion

In conclusion, with regard to the application of bacteria, biopriming application can be used effectively in the of *S. rebaudiana* germination. Based on the promotion of plant growth, microbial inoculants have great potential for sustainable crop production and eco-friendly environmental management

4. References

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